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Molecular Epidemiology of *Cryptococcus neoformans* in Brazil and the United States: Evidence for both Local Genetic Differences and a Global Clonal Population Structure

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Little is known about the global molecular epidemiology of the human pathogenic fungus *Cryptococcus neoformans*. We studied 51 clinical and environmental (pigeon excreta) isolates from two cities in Brazil (Belo Horizonte and Rio de Janeiro) by analyzing their carbon assimilation patterns, electrophoretic karyotypes, restriction fragment length polymorphisms (RFLPs) with the *C. neoformans* repetitive element-1 (CNRE-1), and *URA5* sequences. Results were compared to those previously obtained for isolates from New York City by the same DNA typing methods. Computer-assisted analysis of RFLPs and contour-clamped homogeneous electrophoresis (CHEF) patterns and *URA5* sequences was performed to generate dendrograms. Some environmental and clinical isolates were found to be indistinguishable by CHEF, CNRE-1 RFLP, and *URA5* sequence analyses. Similarly, some isolates from Rio de Janeiro and Belo Horizonte were indistinguishable by the three DNA typing techniques. Overall, Brazilian isolates appeared to be less heterogeneous by DNA analysis than isolates from other regions. Several Brazilian isolates were highly related to New York City isolates. Phylogenetic analysis of the sequences obtained for the Brazilian isolates and those obtained for New York City isolates was congruent with the dendrogram generated from the CNRE-1 RFLP data. In summary our results indicate (i) that the discriminatory power of the DNA typing method differs for Brazilian and New York City strains, with the order being CNRE-1 RFLP analysis > *URA5* sequence analysis > CHEF analysis and CHEF analysis > *URA5* sequence analysis > CNRE-1 RFLP analysis, respectively; (ii) that there are differences in local genetic diversity for Brazilian and New York City isolates; (iii) that there is additional evidence linking clinical isolates to those in pigeon excreta; and (iv) that some isolates from Brazil and New York City are closely related, consistent with the global dispersal of certain pathogenic strains.

Cryptococcus neoformans is an encapsulated yeast which causes life-threatening infections in approximately 5 to 10% of patients with AIDS (10, 44). In Brazil, 4.3% of AIDS-related infections are caused by *C. neoformans* (26), but this number is likely to be an underestimate because cryptococcosis is not a reportable disease. In AIDS patients, meningoencephalitis is the most common clinical manifestation of *C. neoformans* infection, and it is usually incurable, despite antifungal therapy (44). AIDS patients with cryptococcosis who survive the initial presentation are treated with lifelong suppressive antifungal therapy to reduce the likelihood of recurrent infection. DNA typing analysis of initial and relapse isolates obtained from patients with recurrent cryptococcal meningoencephalitis consistently reveals persistence of the same strain, despite antifungal therapy in the majority of patients (4, 37).

Genetic differences among *C. neoformans* strains have been detected by several typing methods, including restriction fragment length polymorphism (RFLP) analysis (11, 12, 40), electrophoretic karyotyping (12, 29), allele sequencing (8), multilocus enzyme electrophoresis (2, 3), and random amplified polymorphic DNA analysis (2, 26a, 37a, 43). Each of these techniques has shown marked genetic heterogeneity among clinical and environmental isolates of *C. neoformans*, even within small geographic areas. For example, Currie et al. (11) defined 18 strains among 25 environmental and clinical *C. neoformans* var. *neoformans* isolates from a single New York City borough. Yamamoto et al. (43) used RAPD analysis to study the epidemiology of environmental and clinical isolates of *C. neoformans* in Nagasaki, Japan, and described six patterns among 21 clinical isolates and three patterns among 8 environmental isolates. The existence of genetic variation for a pathogen is important because it could translate into differences in virulence or response to therapy, and therefore is a consideration in vaccine design. Furthermore, knowledge of genetic variation is essential for understanding the population structure of a microorganism (38).

Despite the increasing number of published studies on the molecular epidemiology of *C. neoformans* in the United States, Europe, Australia, and Japan, there is a noticeable absence of information about the molecular epidemiology of cryptococcal infection in tropical regions. The only study that analyzed isolates from tropical regions is that of Varma et al. (40), who included 20 African isolates in a recent analysis of 156 *C. neoformans* strains from different geographic areas. In Brazil the information on the epidemiology of cryptococcosis is limited to clinical studies (32, 33). In this study we used multiple molecular typing techniques to investigate the molecular epidemiology of *C. neoformans* infection in two Brazilian cities and compared the results with data from New York City isolates. Our results suggest significant differences between the molecular epidemiology of *C. neoformans* infection in tropical and temperate regions.

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General Meeting of the American Society for Microbiology, New Orleans, La., 19 to 23 May 1996 [14a].)

MATERIALS AND METHODS

***C. neoformans* isolates.** Fifty-one *C. neoformans* isolates from Brazil were studied. The isolates originated from two cities, Belo Horizonte and Rio de Janeiro, located in the southeastern region of Brazil and separated by a distance of 400 km. Thirty-three isolates from Belo Horizonte were from AIDS patients (isolates C1 to C30) and three isolates were from non-AIDS patients (isolates C31 to C33). Of the latter three isolates, two were from renal transplant recipients (isolates C31 and C32) and one was from a patient with no identifiable immunodeficiency (isolate C33). Isolates RJ1 to RJ3 were from AIDS patients from Rio de Janeiro. All clinical isolates were recovered from cerebrospinal fluid. All except three of these isolates were obtained from different patients during the initial diagnosis (not relapse) of cryptococcal infection. For three patients who relapsed with cryptococcal infection, the initial and relapse isolates were available for DNA typing.

Fifteen environmental isolates were obtained from pigeon droppings from Belo Horizonte (isolates E1 to E14) and from Rio de Janeiro (isolate RJ4). To recover *C. neoformans* from pigeon excreta, approximately 1.0 to 2.0 g of desiccated excreta from each sample was added to 10 ml of sterilized saline containing penicillin (150 mg/liter) and streptomycin (150 mg/liter). Samples were vortexed and allowed to stand for 5 min. They were then vortexed again, and the suspension was allowed to stand for 30 min to permit settling of the heavier material. Aliquots of 100 μl of the supernatant were spread onto bird seed agar plates supplemented with biphenyl (1.0 g/liter) to inhibit molds. All plates were incubated at 37°C for 21 days, and brown colonies were harvested from positive plates and subsequently streaked to a single colony on a Sabouraud dextrose agar plate. Thus, each positive environmental site was represented by a single colony. Fourteen *C. neoformans* isolates were obtained from 44 locations sampled in Belo Horizonte. The four isolates from Rio de Janeiro were generously provided by B. Wanke (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil). All clinical and environmental isolates were obtained over a 1-year period.

All clinical and environmental isolates were identified as *C. neoformans* by brown colony color in bird seed agar, positive urease test, negative nitrate test, and ability to grow at 37°C (24). All isolates were analyzed for their ability to assimilate 22 carbon compounds, with assimilation testing performed in Wickerham's yeast nitrogen base (1). All isolates produced black colonies in L-dopa agar and were *C. neoformans* var. *neoformans* on the basis of no color change on canavanine-glycine-bromothymol blue agar (25). Biochemical serotyping was performed by growing the isolates on creatinine dextrose bromothymol blue thymine medium, which allows for the grouping of isolates into serotype A or D on the basis of their ability to assimilate thymine (20).

Electrophoretic karyotyping. Karyotype analysis was done by contour-clamped homogeneous electrophoresis (CHEF). *C. neoformans* chromosomal DNA plugs were prepared from cells grown overnight at 30°C in Sabouraud dextrose broth by a modification of published protocols (28, 30). Protoplasts were generated by digesting cells with 10 mg of NovoZym 234 (Novo Biolabs, Bagsvaerd, Denmark) per ml in 1.0 M sorbitol–0.1 M sodium citrate (pH 5 to 6) for 3 h at 30°C. Protoplast-agarose plugs were made by mixing a protoplast suspension (4×10^8 to 6×10^8/ml) with 2% low-melting-temperature agarose solution (Bio-Rad, Richmond, Calif.) to yield a final agarose concentration of 0.66%. Plugs were allowed to harden at 4°C and were then incubated overnight at 50°C in a solution of 1 mg of proteinase K (Boehringer Mannheim, Indianapolis, Ind.) per ml, 1% sarcosine, and 0.1 M EDTA–0.010 M sodium citrate (pH 8.0). The plugs were then washed four times in 50 ml of wash buffer (0.05 M EDTA, 0.020 M Tris-HCl [pH 8.0]) by incubating them at room temperature in wash buffer for 1 h per wash. After a final wash (0.1× wash buffer, 1 h), the plugs were stored at 4°C until used. For electrophoretic karyotyping, the plugs were inserted into a 1% pulsed-field-certified agarose (Bio-Rad) gel (12 by 14 cm), and electrophoresis was performed in a CHEF DR-III variable-angle pulsed-field electrophoresis system (Bio-Rad) in 0.5× TBE buffer (1× TBE is 89 mM Tris-HCl, 89 mM boric acid, and 2 mM EDTA [pH 8.3]) at 14°C. The system was equipped with a Cooling Module (Bio-Rad) for constant temperature control. Electrophoretic conditions were as follows: pulse intervals of 90 s for 9 h, followed by a linear ramp from 120 to 360 s for 39 h at 3.5 V/cm. The gels were stained with ethidium bromide and photographed. Chromosomal DNA plugs from 14 clinical New York City strains were generously donated for comparative analysis by B. Fries (Albert Einstein College of Medicine, Bronx, N.Y.).

DNA isolation. *C. neoformans* DNA was isolated by using a modification of an existing protocol (8). Briefly, protoplasts were made as described above and were then lysed in a solution of 5 mM EDTA, 10 mM sodium citrate, and 1% sarcosine. After 40 min at 65°C, the lysate was extracted twice with buffer-equilibrated phenol and once with chloroform. DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethyl alcohol and was resuspended in 0.01 M Tris-hydrochloride–0.01 M EDTA (pH 8.1).

RFLP analysis. All isolates were typed by Southern blot analysis with the *C. neoformans* repetitive element-1 (CNRE-1), generously provided by Silvia Spitzer and Eric Spitzer (Stony Brook, N.Y.). Genomic DNA was digested with *Sst*I (Sigma Chemical Co.), and the resulting fragments were resolved on a 1% agarose gel and transferred to a positively charged nylon membrane (Boehringer Mannheim) by using a solution of 1.2 M NaCl and 0.5 M NaOH. After UV cross-linking, the membranes were probed with CNRE-1 labeled with [α-^{32}P]dCTP. Hybridization was done in a solution of denatured salmon sperm DNA (0.75 mg/ml) in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 7% sodium dodecyl sulfate (SDS), 10× Denhardt's reagent, and 5% dextran sulfate in 0.020 M sodium phosphate buffer (pH 7.2) at 65°C. Filters were washed with 3× SSC–5% SDS–10× Denhardt's reagent and 1× SSC–1% SDS at 65°C. Bands were visualized by autoradiography.

PCR and *URA5* RFLPs. The 779-bp *URA5* gene (coding for orotidine monophosphate pyrophosphorylase) (13) was amplified from genomic DNA by PCR with the oligonucleotides TTAAGACCTCTGAACACC and ATGTCCTCCCAAGCCCTC as described previously (8). PCR conditions were 92°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min for 35 cycles. PCR products were analyzed by agarose gel electrophoresis and identified as *URA5* DNA by Southern blotting with a [γ-^{32}P]dATP-labeled oligonucleotide (TCGTCCCTTGAGTGGCGC) complementary to an internal *URA5* sequence. The amplified *URA5* DNA for each isolate was digested with *Hae*II, *Hin*dIII, *Fok*I, *Bst*XI, *Hae*III, *Stu*I, and *Msp*I (Boehringer Mannheim) in separate reactions. Restriction enzyme fragments were separated in a 1 to 1.2% agarose gel.

DNA sequencing. PCR-amplified *URA5* DNA from 14 different isolates including 4 from human immunodeficiency virus (HIV)-positive patients from Belo Horizonte (isolates C5, C7, C24, and C25), 2 from HIV-positive patients from Rio de Janeiro (isolates RJ1 and RJ2), 2 from HIV-negative patients (isolates C31 and C33), and 6 environmental isolates (isolates E3 to E6, E9, and E12) were sequenced. To do this, each PCR product was cloned into the pCR 2.1 vector of the TA cloning system (Invitrogen, San Diego, Calif.). *Escherichia coli* transformants were selected on plates containing 50 μg of kanamycin per ml, and plasmid DNA was purified with Maxi-prep columns (Qiagen, Chatsworth, Calif.). The insert was sequenced in the DNA Sequencing Facility of the Albert Einstein College of Medicine with automated sequencing instrument models ABI373A and ABI377 (Perkin-Elmer, Foster City, CA). Samples were analyzed by fluorescent cycle sequencing with dye-labeled primers.

Data analysis. Prior to the computer-assisted analysis described below, the karyotype and CNRE-1 RFLP patterns of all strains were compared by visual inspection. Patterns were considered identical if all bands matched exactly. Patterns that differed by only one band were identified as subtypes (indicated as "a" and "b" in Fig. 5). Isolates that differed from the others by at least two bands were assigned to different patterns.

Karyotype and CNRE-1 RFLP patterns were compared by using the Molecular Analyst/PC Fingerprinting software (Bio-Rad, Hercules, Calif.). CHEF pictures and CNRE-1 RFLP patterns were scanned into the instrument database by using a Gel Doc 1000 scanner (Bio-Rad). The CNRE-1 RFLP patterns of New York City clinical and environmental isolates reported earlier (11) were also scanned and compared to those of the Brazilian isolates. The RFLP patterns were normalized by equating the *Hin*dIII-digested bacteriophage λ DNA molecular weight markers (Boehringer Mannheim). Similarly, *Saccharomyces cerevisiae* chromosomal DNA (Bio-Rad) was used in a lane as a reference to normalize the CHEF patterns. Band positions were automatically identified, and Dice coefficients of similarity (number of shared bands × 2 × 100/total number of fragments in the two samples) were determined for each pair of strains compared, generating a matrix of similarity coefficients. When two patterns were compared, a match was recorded if the normalized molecular size of the fragment in the first pattern was within a window of ±5% of the molecular size of a fragment in the second pattern. Dendrograms based on the similarity coefficients were then generated for selected strains by the unweighted pair-group method of average linkage (UPGMA) (31).

To estimate phylogenies, *URA5* sequences obtained from the Brazilian isolates were first aligned with those obtained for seven American isolates (9), isolate B-3501 (13), and *C. neoformans* var. *gattii* (7) by the program Clustal V (18). *URA5* sequences for American isolates (sequences L38582, L38583, L38584, L38585, L38586, L38587, and L38588), isolate B-3501 (sequence M34606), and *C. neoformans* var. *gattii* (sequence M93026) were downloaded from GenBank. Trees were inferred with the DNAPENNY (branch and bound parsimony) and DNAML (maximum likelihood) programs of the PHYLIP package, version 3.5c (14). The data set was also bootstrapped (100 replicates) by sequential use of SEQBOOT, DNAPENNY, and CONSENSE of the PHYLIP package, version 3.5 (14). Sequences from the Brazilian isolates were also manually compared to another set of partial *URA5* sequences from New York City isolates J2 and J3 (8).

The discriminatory power of each typing technique was determined by calculating the discriminatory index (*D*) of Hunter and Gaston (19). This index represents the probability that two unrelated strains will be assigned to different types by a given typing method. *D* is calculated by the following formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j(n_j - 1)$$

where N is the total number of strains in the sample population, s is the total number of types observed, and n_j is the number of strains belonging to the jth type.

TABLE 1. Carbon assimilation patterns for 51 *C. neoformans* isolates

Pattern	Carbon source[a]						No. of isolates with pattern	CNRE-1 pattern[b]
	D-Arabinose	L-Arabinose	Raffinose	D-Ribose	Succinic acid	Citric acid		
1	+	+	+	+	+	+	24	A, B, C, D, H, I, K, L
2	+	+	+	+	−	+	11	A, B, C, F, J
3	+	+	+	+	+	−	4	B, C, L, T
4	+	−	+	+	+	+	4	C, F, J, S
5	+	+	+	+	−	−	3	A, B, R
6	+	+	−	−	−	+	2	N, P
7	−	−	+	+	+	+	1	Q
8	+	−	+	+	+	−	1	M
9	+	−	+	+	−	+	1	K

[a] A positive sign means assimilation; a negative sign means inability to use a compound as a sole carbon source.
[b] Patterns as defined in Fig. 5.

Statistical analysis. The distributions of the CHEF and CNRE-1 RFLP patterns were compared by chi-square analysis. The Student *t* test was used for pairwise comparisons of the nucleotide sequences and Dice coefficients among Brazilian and New York City isolates. A *P* value of less than 0.05 was considered significant. Statistical analysis was performed by using Primer for Biostatistics: The Program, version 3.01 (25a).

Nucleotide sequence accession numbers. The *URA5* DNA sequences of isolates C7 (same as isolates C31, E5, and RJ2), C5 (same as isolate C33), C24, C25, E3, E4, E6, E9, E12, and RJ1 have been deposited in GenBank under accession numbers U67723, U67724, U67725, U67726, U67727, U67728, U67729, U67730, U67731, and U67732, respectively.

RESULTS

Biochemical characterization. All *C. neoformans* strains are able to assimilate glucose, sucrose, galactose, maltose, cellobiose, trehalose, xylose, rhamnose, erythritol, ribitol, D-mannitol, inositol, and melezitose but do not assimilate lactose, melibiose, or glycerol. By these criteria all isolates were typed as *C. neoformans*. However, isolates varied in their abilities to assimilate raffinose, L-arabinose, D-arabinose, D-ribose, succinic acid, and citric acid. On the basis of the carbon assimilation profiles, the 51 isolates were grouped into nine groups according to their distinguishable carbon assimilation patterns (Table 1). Biochemical classification in creatinine-dextrose-bromothymol blue-thymine (20) grouped 80% of the isolates to serotype A and 20% to serotype D (data not shown).

Electrophoretic karyotyping. CHEF analysis of the 51 Brazilian clinical and environmental isolates of *C. neoformans* revealed 14 distinguishable karyotype patterns. Representative karyotype patterns are illustrated in Fig. 1. Forty-one percent of the isolates fell into a single karyotype pattern (pattern I), and eight patterns contained only one isolate each (see Fig. 4). The average number of chromosomes was 10.8 ± 0.9, and the chromosome number ranged from 10 to 13. Among isolates assigned to each of patterns I, II, and IV to VI there were both clinical and environmental isolates from Belo Horizonte. Furthermore, patterns I and II included isolates from Rio de Janeiro that had karyotypes apparently identical to those of both clinical and environmental isolates obtained from Belo Horizonte. Initial and relapse isolates from individual patients had identical CHEF patterns. Some isolates from HIV-positive and HIV-negative patients also had indistinguishable CHEF patterns. We also compared karyotype patterns between Brazilian and 14 New York City isolates and found that the patterns of three New York City clinical isolates showed high degrees of similarity to patterns I (S = 78%), IV (S = 86%), and VI (S = 94%), respectively.

CNRE-1 RFLPs. CNRE-1 RFLP analysis revealed 20 different patterns (patterns A to T) among the 51 isolates, with each pattern having from 14 to 20 DNA hybridization bands. Representative CNRE-1 RFLP patterns are illustrated in Fig. 2. Patterns A, B, and C were highly related (see Fig. 5) and included 43% of the isolates. Patterns A, C, D, F, J, and K each included clinical and environmental isolates that had indistinguishable fingerprints by RFLP analysis. Similarly, the CNRE-1 RFLPs of some isolates from Rio de Janeiro were indistinguishable from those of Belo Horizonte isolates (patterns A and H). Initial and relapse isolates from individual patients had identical CNRE-1 RFLP patterns. No pattern type was associated with a particular patient group.

***URA5* sequencing and RFLPs.** Digestion of PCR-amplified *URA5* DNA with *Bst*XI, *Msp*I, *Stu*I, and *Hae*III revealed identical RFLP patterns for the 51 Brazilian isolates (data not shown). This was surprising because *URA5* is highly polymorphic in North American isolates, and we proceeded to determine the *URA5* sequences of 14 clinical and environmental isolates from Brazil. Sequence analysis revealed the absence of *Hae*II, *Hin*dIII, and *Fok*I restriction sites in Brazilian isolates, even though restriction sites for these enzymes were common in North American isolates (8).

The 779-bp coding sequences of the *URA5* gene obtained for 14 isolates are presented in Fig. 3, along with the sequences obtained for strains B-3501 (13) and J2 (8). The *URA5* sequences from the 14 Brazilian isolates were highly homologous and were closely related to the sequence of New York City isolate J2. Brazilian isolates C7, C31, E5, and RJ2 had identical

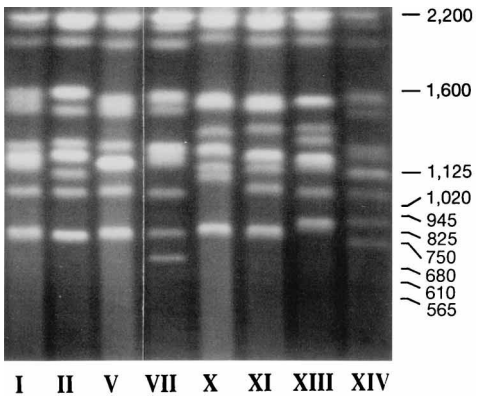

FIG. 1. Representative electrophoretic karyotypes illustrating several of the patterns obtained from Brazilian *C. neoformans* isolates. Pattern identifications are indicated as roman numerals (see Fig. 5). Chromosomal DNA molecular size markers are indicated along the right margin.

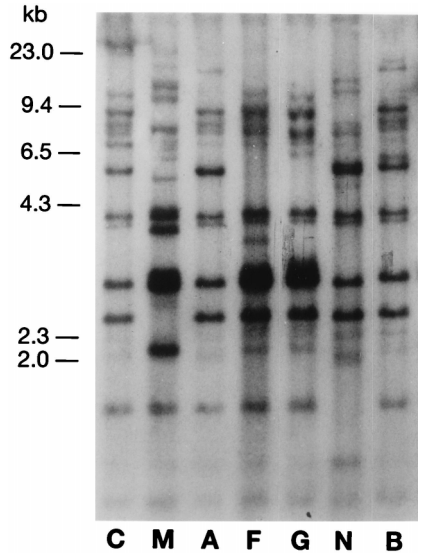

FIG. 2. Representative CNRE-1 RFLPs illustrating several patterns obtained from Brazilian *C. neoformans* isolates. Pattern identifications are indicated as capital letters (see Fig. 6). DNA molecular size markers are indicated along the left margin.

sequences, as did isolates C5 and C33. The remaining isolates, with the exception of isolates C25 and E3, differed at most by three positions from those mentioned above. The gene from strain E3 showed the greatest number of base substitutions, amounting to 1.2% of the determined sequence, when compared to the number among the other Brazilian isolates studied. It was previously shown that isolates J2 and ATCC 24064 (serotype A) had identical sequences, and the sequences of both strains differed by a single base from that of isolate J3 (8). Brazilian isolates C7, C31, E5, and RJ2 had identical sequences compared to the available sequence of J2 (nucleotide positions 40 to 744) (8). Moreover, a single base change was observed between J2 and Brazilian isolates C24, E4, E6, E12, and RJ1. Isolate E12 showed the same sequence as New York City isolate J3 (8). However, the genes from strain J2 (and J3) and the Brazilian isolates had many base differences from that of B-3501, although most differences were found within introns or in the third position of the codon (silent substitutions). The amino acid sequences of strains C5, C7, C24, E4, E9, and E12 were identical, whereas at most 2 amino acid substitutions (from those isolates cited above) were detected in the amino acid sequences of the other isolates. Overall, the numbers of *URA5* base differences (average ± standard deviation) among 14 Brazilian and 10 New York City isolates were 3.8 ± 3.4 (n = 91) and 15.6 ± 9.8 (n = 45), respectively ($P < 0.001$; Student's t test). The analysis of nucleotide sequences for the 10 New York City isolates was based on pairwise comparisons of previously published sequencing data (8, 9).

Computer-assisted analysis of CHEF and CNRE-1 RFLP patterns and *URA5* sequences. Percent similarities based on the Dice coefficient and UPGMA clustering for the 14 CHEF patterns are presented in Fig. 4. The similarity for the different patterns ranged from 26 to 93%. The dendrogram revealed four main clusters with more than 57% similarity (clusters 1a, 2, 3, and 4) and five subclusters with similarities ranging from 71 to 93%. Subcluster 3b contained 41% of the isolates with 86% similarity. Figure 5 shows a dendrogram constructed from the Dice coefficients for the 20 CNRE-1 RFLP patterns. Similarities among the different CNRE-1 patterns ranged from 73 to 96%. The dendrogram in Fig. 5 shows five major clusters of patterns with more than 80% similarity. Five subclusters with more than 90% similarity could also be identified (subclusters 1a, 2a, 2c, 3b, and 4a). Among those, subcluster 2a contained 43% of the isolates. The computer-assisted analysis of CHEF and CNRE-1 patterns was in good agreement with manual alignment of patterns on the basis of visual analysis.

To compare the genetic relatedness among Brazilian and New York City isolates, we generated a mixed dendrogram based on similarity coefficients computed between the RFLP patterns obtained for Brazilian isolates and 13 clinical isolates (isolates J10, J12, J15, J16, J19, J20, J21, J22, J24, and J26) and environmental New York City isolates (isolates B5, B6, and B7) (described previously [9]). In this dendrogram (Fig. 6) several representative patterns computed for the Brazilian isolates remained intact in clusters. There was, however, penetration of some New York City strains into four subclusters of Brazilian isolates with at least 90% similarity. Remarkably, the pattern of New York City isolate B7 showed 100% similarity with pattern D of the Brazilian isolates.

Parsimony analysis of the *URA5* sequences revealed a consensus tree in which the majority of Brazilian and New York City isolates clustered separately (Fig. 7). However, there was penetration of New York City isolates J2 and J3 into clusters of Brazilian strains, as observed in the CNRE-1 RFLP analysis (Fig. 6). We found the same pattern of clustering for the New York City isolates as the one described in the study of Chen et al. (9). Use of maximum likelihood analysis gave a tree that was consistent with the one obtained by parsimony analysis (data not shown). For some isolates the trees derived by CNRE-1 RFLP and *URA5* analyses were in general agreement, as demonstrated by the fact that both analyses yielded the same or highly related clusters of isolates. For example, isolates C25 and E3 were grouped together according to their *URA5* sequences and had the same CNRE-1 RFLP pattern. A similar association was found for isolates E9 and E12 (pattern D) and isolates C5 and C33 (patterns B and C, respectively). Furthermore, some strains which were grouped on the basis of highly similar or identical CNRE-1 RFLP patterns also had the same or highly related CHEF pattern. For example, strains C12 and E2 (pattern F) were clustered together with strain E4 (pattern G) on the basis of their RFLP patterns, and these three strains had the same CHEF pattern (pattern V).

Determination of *D* for each typing technique. The *D* values of biotyping by carbon assimilation, electrophoretic karyotyping, CNRE-1 RFLP analysis, and *URA5* sequencing are presented in Table 2, along with the *D* values obtained for some of the same techniques when applied to New York City isolates (calculated on the basis of data presented previously [8, 9]). For the Brazilian isolates the *D* values for CNRE-1 RFLP analysis and *URA5* sequencing were high, followed by karyotyping and biotyping. For New York City isolates CHEF analysis was the most discriminatory typing system.

DISCUSSION

The 51 Brazilian isolates included in this study were all *C. neoformans* var. *neoformans*. This is consistent with reports that *C. neoformans* var. *neoformans* is prevalent among immunosuppressed patients (mainly AIDS patients) not only from Brazil (32, 33) but also from all over the world (27). Also, this variety has long been associated with pigeon excreta, the source of the environmental isolates in this study (27). The majority of the isolates examined were serotype A, consistent with the predominant serotype isolated in most countries. The in vitro susceptibilities of the 51 isolates to amphotericin B,

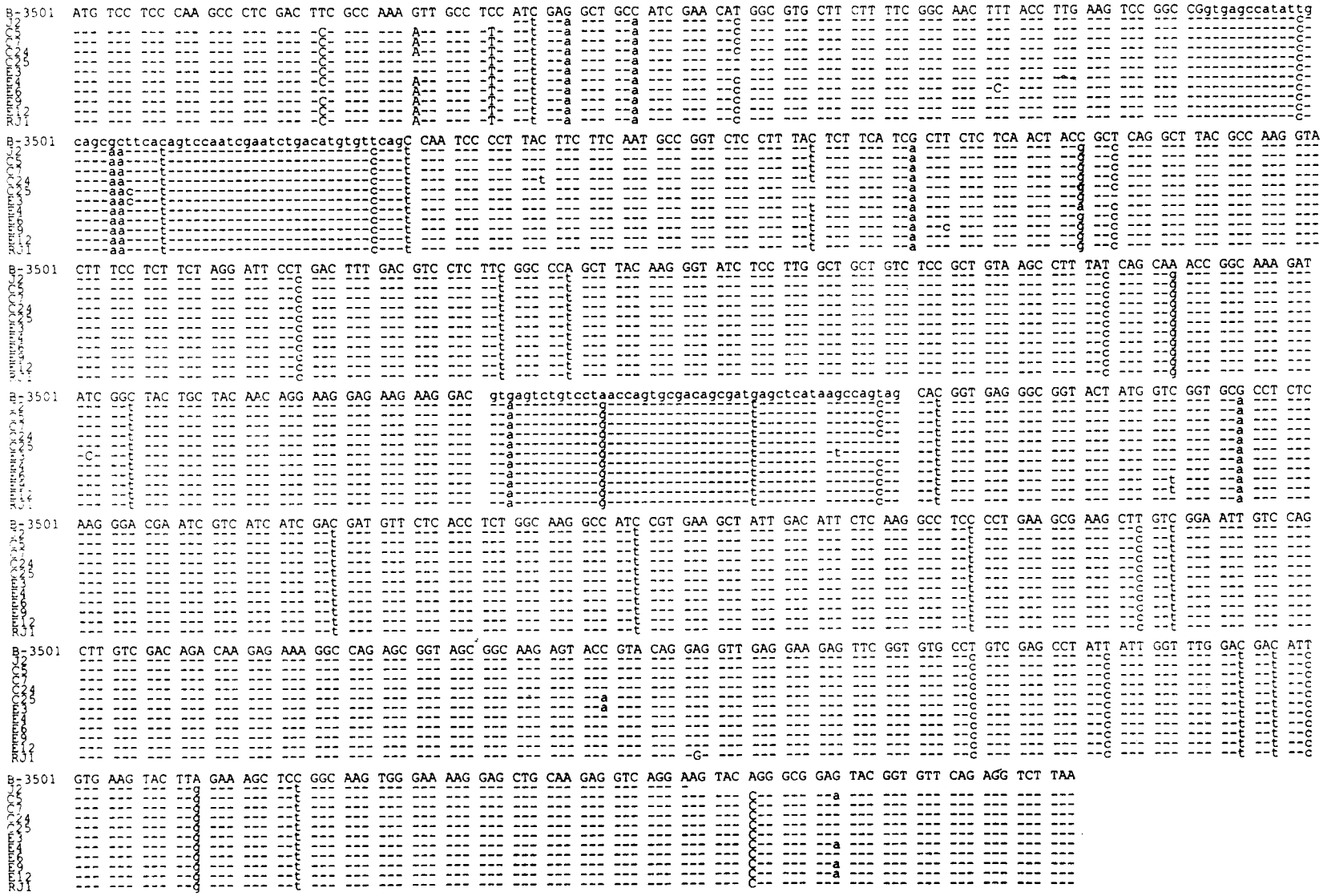

FIG. 3. Nucleotide sequence of the *URA5* genes from isolates B-3501 (13), J2 (8), C5, C7, C24, C25, E3, E4, E6, E9, E12, and RJ1. A hyphen indicates that the base is identical to that in B-3501. A space implies that the base was not present in the allele. Lowercase letters indicate synonymous substitutions. Capital letters indicate nonsynonymous substitutions. The first and last 18 bases in the sequence of each isolate corresponds to the *URA5*-specific amplification primers.

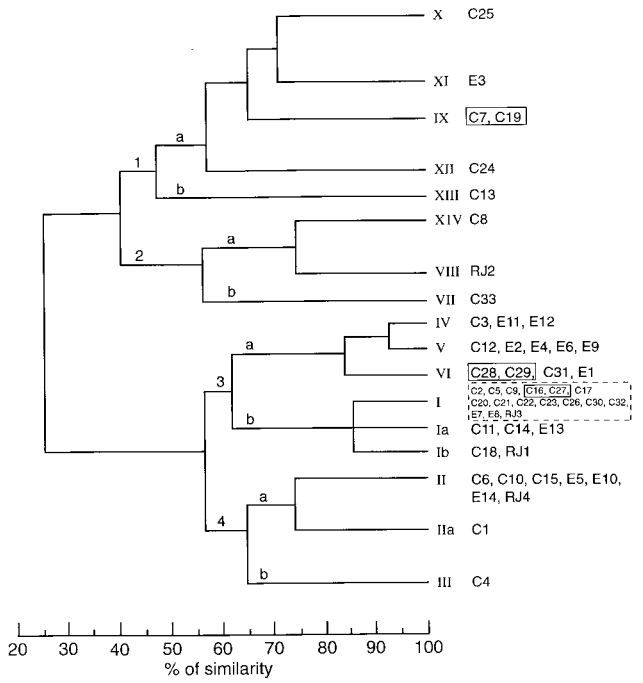

FIG. 4. Dendrogram generated from the Dice coefficients computed from the CHEF patterns (patterns I to XIV) for 51 clinical and environmental *C. neoformans* isolates. Isolates are listed on the right. Boxes represent initial and relapse isolates from an individual patient. Vertical lines connect groups with the indicated percent similarity. Arabic numbers indicate major groups, lowercase letters indicate subgroups, and roman numerals designate pattern names.

flucytosine, ketoconazole, fluconazole, and itraconazole have been determined, and the results revealed similar MICs for all isolates (15). Hence, the Brazilian isolates are phenotypically similar to those isolated from other parts of the world.

Three DNA typing techniques were used to study the molecular epidemiology of the Brazilian isolates: electrophoretic karyotyping, CNRE-1 RFLP analysis, and *URA5* sequence analysis. Electrophoretic karyotyping has been shown to be a useful technique for distinguishing *C. neoformans* isolates because there is great variation in the size and number of chromosomes among cryptococcal isolates (12, 16, 27, 29, 42). The number of electrophoretic karyotype patterns among the Brazilian isolates was lower than the number in previous studies of North American (29) or European (12) isolates. For example, we found only 14 karyotype patterns among 51 isolates, whereas Perfect et al. (29) found 41 patterns among 46 isolates ($P < 0.001$; chi-square analysis) and Dromer et al. (12) found 39 patterns among 40 isolates ($P < 0.001$; chi-square analysis). Differences in CHEF technique do not account for this difference since a recent study from our laboratory in which the same methods and equipment were used found extensive karyotype diversity among isolates from two closely located hospitals in New York City (16). *S* values for New York City and Brazilian isolates were 25% ± 6% ($n = 44$) and 44% ± 15% ($n = 153$) ($P < 0.001$; *t* test). Hence, there was less diversity in the electrophoretic karyotypes of the Brazilian isolates than among New York City isolates or other Northern Hemisphere isolates (12, 16, 29).

The CNRE-1 probe is a complex sequence that hybridizes to a family of polymorphic repetitive DNA elements found in all chromosomes of *C. neoformans* (36). CNRE-1 RFLP analysis has been used in previous studies of *C. neoformans* molecular epidemiology and has been found to be highly discriminatory (11, 36, 37). The stability of CNRE-1 RFLP patterns has previously been documented in initial and relapse isolates from human patients (37) and after passage in mice or serial subcultures in Sabouraud dextrose agar (11). Analysis of the Brazilian isolates for CNRE-1 RFLPs revealed 20 patterns among 51 isolates. In contrast, Currie et al. (11) identified 18 CNRE-1 RFLP patterns among 25 New York City isolates ($P = 0.015$; chi-square analysis). CNRE-1 RFLP analysis, like electrophoretic karyotyping, revealed less diversity among Brazilian isolates than among New York City isolates.

The relative homogeneity of the Brazilian isolates was also apparent from the *URA5* sequence and RFLP analyses. Comparison of sequences of *URA5* DNA from 14 Brazilian isolates revealed an average of 3.8 ± 3.4 nucleotide differences for 91 pairwise comparisons. In contrast, analysis of 10 previously determined *URA5* sequences from New York City isolates revealed an average of 15.6 ± 9.8 nucleotide differences for 45 pairwise comparisons. There was a significant difference ($P < 0.001$; *t* test) for the comparison between the number of nucleotide differences found among Brazilian and New York City isolates. Digestion of amplified *URA5* DNA from the 51 Brazilian isolates with seven restriction enzymes revealed no differences in RFLPs. These enzymes had been shown to discriminate among *URA5* genes from New York City isolates (8), but the sequence polymorphisms which produced RFLPs were not found in the *URA5* sequences of the Brazilian isolates. Despite the differences between the *URA5* sequences of Brazilian and New York City isolates, it is noteworthy that there was still a high degree of homology (100%) between some isolates (e.g.,

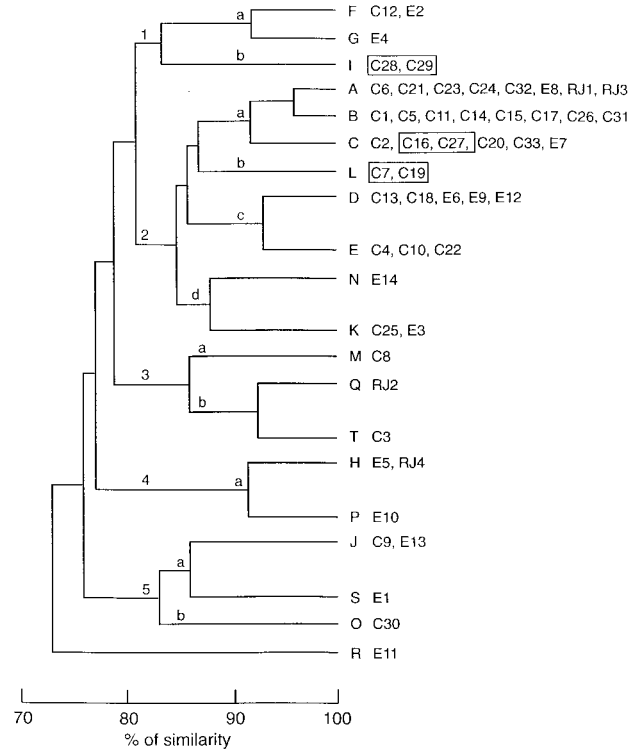

FIG. 5. Dendrogram generated from the Dice coefficients computed from the CNRE-1 RFLP patterns (patterns A to T) for 51 clinical and environmental *C. neoformans* isolates. Isolates are listed on the right. Boxes represent initial and relapse isolates from an individual patient. Symbols and letters are as described in the legend to Fig. 5, except that capital letters designate individual patterns.

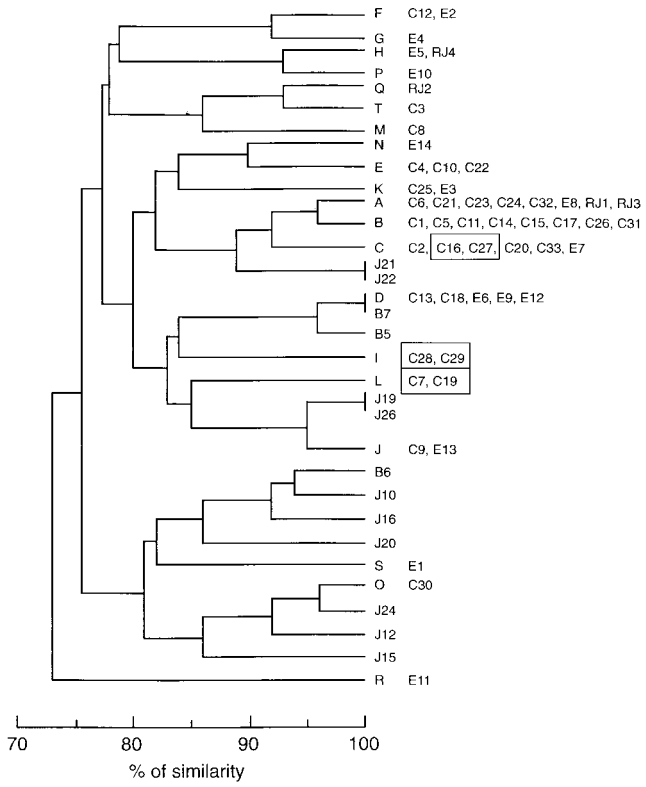

FIG. 6. Dendrogram generated from the Dice coefficients computed from the CNRE-1 RFLP patterns (patterns A to T) for 51 Brazilian isolates and 13 clinical (J10, J12, J15, J16, J19, J20, J21, J22, J24, and J26) and environmental (B5, B6, and B7) New York City isolates. Isolates are listed on the right. Boxes represent initial and relapse isolates from an individual patient. Symbols and letters are as described in the legend to Fig. 5. Note that New York City isolates B5, B7, J19, and J26 clustered into groups of Brazilian isolates with more than 90% similarity.

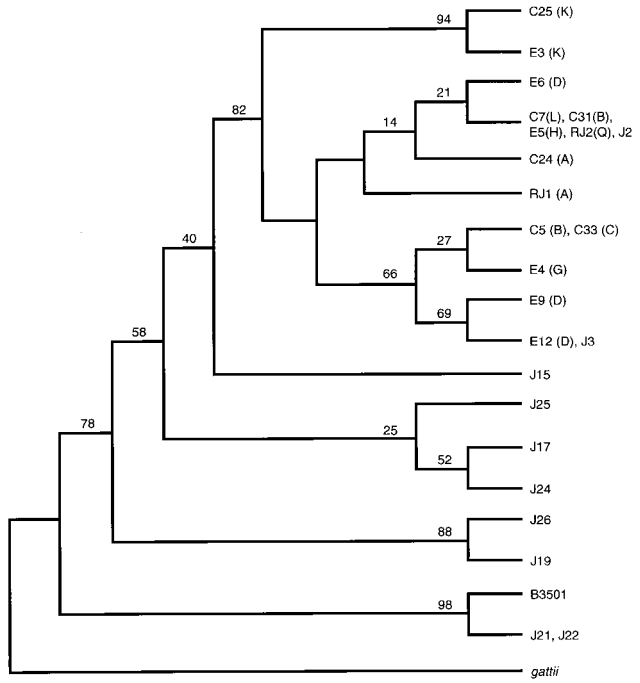

FIG. 7. Relationship of *C. neoformans* isolates obtained from phylogenetic analysis of *URA5* sequence data. The CNRE-1 patterns are listed in parentheses after the name of each isolate. Numbers at the branch points indicate the number of times that the group consisting of the isolates which are to the right of that fork occurred among 100 bootstrap replications. *C. neoformans* var. *gattii* was designated the outgroup. The J2 and J3 strains were added to the dendrogram manually. The J2 sequence has 100% homology to the sequences of isolates C7, C31, E5, and RJ2. The J3 sequence has 100% homology to the sequence of isolate E12. The J2 and J3 sequences were not used in the computer analysis to generate dendrograms because small segments of 5′ and 3′ sequences were not available (8).

isolate J2 versus isolates C5, C7, C31, C33, E5, and RJ2 and isolate J3 versus isolate E12).

The three DNA typing techniques used in this study each revealed less diversity among Brazilian isolates than among New York City isolates. The factors responsible for the differences in genetic diversity between Brazilian and New York City isolates are not understood. Differences in climate may account for the greater diversity among New York City isolates, but the mechanism by which such diversity is generated and maintained is not known. For Brazilian and New York City isolates the most discriminatory techniques were CNRE-1 RFLP and *URA5* sequence analyses, respectively (Table 2). Biotyping by carbon assimilation patterns exhibited the lowest *D* values, with no obvious correlation to DNA typing evident in our isolate set. Despite the higher *D* values by the CNRE-1 RFLP technique than by CHEF analysis, the latter method produced lower *S* values, and consequently suggested less genetic relatedness among strains than did CNRE-1 RFLP analysis (Fig. 4 and 5). Previous studies have shown karyotype polymorphisms, despite identical or highly related CNRE-1 RFLP patterns for some isolates (4, 9). Chromosomal rearrangements are common in *C. neoformans*, and karyotype instability could explain the lower *S* values calculated for the CHEF data (16).

The finding that isolates obtained from pigeon excreta and from patients were closely related adds to the increasing evidence linking clinical isolates to those found in pigeon excreta. Evidence for an epidemiological link between the habitat of *C. neoformans* and human cryptococcosis has been suggested for both *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* by different molecular typing methods (11, 22, 35, 43). No differences in karyotype patterns, RFLP patterns, or results of sequencing analysis were observed when isolates from HIV-infected patients were compared to those from HIV-negative patients. However, the number of isolates from HIV-negative patients was small and analysis of a larger number of isolates is necessary before definitive statements can be made on this subject. In addition, initial and relapse isolates had identical CHEF and CNRE-1 RFLP patterns, consistent with previous findings that the majority of cases of recurrent cryptococcal

TABLE 2. *D* values by different typing techniques applied for Brazilian and New York City *C. neoformans* isolates

Typing technique	*D* value	
	Brazilian[a]	NYC[b]
Biochemical	0.73	NA[c]
CHEF analysis	0.87	1.00
CNRE-1 RFLP analysis	0.93	0.95
URA5 sequencing	0.92	0.99

[a] *D* values for Brazilian isolates were calculated from data in this report.
[b] *D* values for New York City isolates were calculated from data presented elsewhere (6, 9, 13).
[c] NA, not available.

meningitis result from the persistence of the initial infection rather than reinfection with newly acquired strains (4, 29, 37, 40).

Calculation of pattern similarity and generation of dendrograms based on *S* values provides a method for visualizing clusters of related strains, especially in studies involving large samples or retrospective comparisons (34). Also, phylogenetic analyses are ideally suited for addressing the issue of recombination versus clonality (5). Clonal populations evolve in a tree-like fashion and support short and well-resolved trees, whereas recombining populations do not fit well to phylogenetic trees since their evolutionary pattern is more like a net (5, 6). As seen in Fig. 7, the consensus tree generated from *URA5* sequencing data is short and well resolved and hence is consistent with a clonal population structure. In addition, the penetration of New York City isolates into clusters of Brazilian isolates in the dendrograms generated with the CNRE-1 RFLP analysis and *URA5* sequencing data (Fig. 6 and 7) supports the notion that the highly related groups of Brazilian and New York City isolates are clonally related. Genetic concordance between Brazilian and New York City isolates was also demonstrated by CHEF analysis, again suggesting that these isolates are clonally related. The presence of a particular genotype in great excess (especially when the genetic information derives from high-resolution methods such as DNA sequencing and RFLP analysis) is a significant indication of clonal reproduction (38, 39). Our findings are consistent with this criterion in that one genotype prevailed independently of the analytical method (*URA5* sequencing and CHEF and RFLP analyses), even in geographically distant sites (Belo Horizonte and Rio de Janeiro). Another evidence of clonality is a correlation between independent sets of genetic markers (38, 39). In the present study we found concordance between *URA5* sequencing and CNRE-1 RFLP analysis (some isolates clustered together in dendrograms generated by these two techniques) and between CNRE-1 RFLP analysis and electrophoretic karyotyping. These findings agree with a previous study of New York City isolates by CNRE-1 RFLP analysis and *URA5* sequence analysis which showed that both typing methods yield congruent relationships, consistent with a clonal population structure (9). These findings provide strong support for clonality in a subset of *C. neoformans* strains. In this regard, the *C. neoformans* strains analyzed in this study appear to be different from those of the fungi *Coccidioides immitis* (5) and *Histoplasma capsulatum* (6), which have been reported to have a recombining population structure.

A clonal population structure for *C. neoformans* is consistent with several biological characteristics of this fungus. *C. neoformans* var. *neoformans* exists as two mating types, *a* and α, but sexual reproduction has only been demonstrated under appropriate laboratory conditions for a few strains. However, the overwhelming majority of clinical and environmental isolates are mating type α, suggesting that widespread sexual recombination in natural populations of *C. neoformans* does not occur or is infrequent (23). Furthermore, there is great heterogeneity in chromosome size and number of *C. neoformans* strains, which suggests mating incompatibility. The karyotype heterogeneity in *C. neoformans* strains is in contrast to the findings for *Aspergillus nidulans*, which reproduces sexually and exhibits indistinguishable karyotypes (17). Recently, Wickes et al. (41) demonstrated that α strains of *C. neoformans* can undergo a true dimorphic transition from a haploid yeast phase to a hyphal phase with production of viable basidiospores, all belonging to mating type α. They also proposed that these basidiospores represent the infectious form of *C. neoformans*. The occurrence of haploid fruiting with production of infectious basidiospores (without the occurrence of meiosis) provides another mechanism for clonal reproduction in *C. neoformans.*

Our results demonstrate local geographic differences in the molecular epidemiology of *C. neoformans* that could result from natural selection by specific environmental and climatic conditions at individual geographic sites. The results also suggest that there may be widespread global distribution of certain pathogenic *C. neoformans* strains. *C. neoformans* var. *neoformans* strains are found on all continents in frequent association with avian excreta. Global dispersal of certain clones could occur as a result of wind transport and/or bird migrations. It is noteworthy that the common pigeon (*Columba livia*) was originally found in Europe and North Africa and has subsequently been introduced throughout the world by humans (21). It is conceivable that transplantation of this bird species from the original habitat to the Americas facilitated the spread and establishment of certain *C. neoformans* clones. Clonal relatedness suggests a correlation between genetic and biological characteristics like virulence, drug susceptibility, and antigenic composition. Our results suggesting the existence of widespread clonally related strains provide a starting point for future studies correlating biological properties with individual genetic lineages.

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